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Association of a gene-expression subtype to outcome and treatment response in patients with recurrent/metastatic head and neck squamous cell carcinoma treated with nivolumab

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ABSTRACT

Background Immune checkpoint inhibitors have been approved and currently used in the clinical management of recurrent and metastatic head and neck squamous cell carcinoma (R/M HNSCC) patients. The reported benefit in clinical trials is variable and heterogeneous. Our study aims at exploring and comparing the predictive role of gene-expression signatures with classical biomarkers for immunotherapy-treated R/M HNSCC patients in a multicentric phase IIIb trial.

Methods Clinical data were prospectively collected in Nivactor tiral (single-arm, open-label, multicenter, phase IIIb clinical trial in platinum-refractory HNSCC treated with nivolumab). Findings were validated in an external independent cohort of immune-treated HNSCC patients, divided in long-term and short-term survivors (overall survival >18 and <6 months since the start of immunotherapy, respectively). Pretreatment tumor tissue specimen from immunotherapy-treated R/M HNSCC patients was used for PD-L1 (Tumor Proportion Score; Combined Positive Score (CPS)) and Tumor Mutational Burden (Oncopanel TSO500) evaluation and gene expression profiling; classical biomarkers and immune signatures (retrieved from literature) were challenged in the NIVACTOR dataset.

Results Cluster-6 (Cl6) stratification of NIVACTOR cases in high score (n=16, 20%) and low score (n=64, 80%) demonstrated a statistically significant and clinically meaningful improvement in overall survival in the high-score cases (p=0.00028; HR=4.34, 95% CI 1.84 to 10.22) and discriminative ability reached area under the curve (AUC)=0.785 (95% CI 0.603 to 0.967). The association of high-score Cl6 with better outcome was also confirmed in: (1) NIVACTOR progression-free survival (p=4.93E-05; HR=3.71, 95% CI 1.92 to 7.18) and objective-response-rate (AUC=0.785; 95% CI 0.603 to 0.967); (2) long survivors versus short survivors (p=0.00544). In multivariate Cox regression analysis, Cl6 was independent from Eastern Cooperative Oncology Group performance status, PDL1-CPS, and primary tumor site.

Conclusions These data highlight the presence of underlying biological differences able to predict survival and response following treatment with immunotherapy in platinum-refractory R/M HNSCC that could have translational implications improving treatment selection.

Trial registration number EudraCT Number: 2017-000562-30.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Immune checkpoint inhibitors are currently offered to recurrent and metastatic head and neck squamous cell carcinoma (R/M HNSCC) patients; however, only few patients will benefit of the treatment.

WHAT THIS STUDY ADDS

⇒ Two gene-expression signatures are able to improve patient selection compared with the biomarkers (such as PD-L1 and tumor mutational burden) currently in clinical use.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ There is a great need for effective biomarkers in R/M HNSCC and gene expression prognostic and predictive signatures prove their value to ameliorate patients' clinical management.

BACKGROUND

In recurrent/metastatic (R/M) head and neck squamous cell carcinoma (HNSCC) not amenable to salvage surgery or radiation, the overall survival (OS) remains unsatisfactory. Treatment with immune checkpoint inhibitors (ICIs) has shown survival benefit

in randomized clinical trials both in the setting of platinum pretreated patients and in patients at their first line of treatment,[1–3] transforming the treatment for R/M HNSCC patients. For some patients, long-lasting responses have been observed, however, only a small proportion of patients benefit from immunotherapy and there is an unmet need for predictive markers. Accounting for the HNSCC heterogeneity, several attempts to identify patients who could respond to immunotherapy were applied, but clinical parameters essentially failed. Molecular determinants, such as genomic and gene-expression (GE) signatures, were thus analyzed as potential drivers of response. Several GE microenvironment patterns have been published so far, and an inflamed response was observed in approximately 40% of cases.[4] However, within this group, different immune activities (active or exhausted) were recorded.

Recently, several immune-associated GE signatures have been reported as associated with response to ICI among different tumor types[5–7] and the multilevel combination including tumor mutational burden (TMB), PD-L1 combined positive score, and T-cell inflamed GE profile revealed the ability to predict clinical responses to anti-PD-1 in different tumors including HNSCC.[8] These models often include superimposable biological pathways, so making these gene GE signatures at risk of overlapping between each other. A previous GE HNSCC meta-analysis[9] conducted on public datasets identified an "immune-reactive" (cluster-6, Cl6) subtype, characterized by similarity to normal tissue, overexpression of IFN-I associated genes and good prognosis.

Here, we showed the data obtained with the aim to identify patients who could obtain the greatest benefit from ICI therapeutic option. We applied selected immune-related signatures, also to test the independence or the associations between the previously published model, and other specific biomarkers to a cohort of 80 R/M HNSCC patients (platinum-refractory) enrolled in a prospective trial, named NIVACTOR and we validated this signature in another cohort of 20 patients characterized by short and long-term survival when treated with ICI.

MATERIALS AND METHODS

Study design

The Nivactor GE database was considered as training set (online supplemental etable 1), while for testing set a cohort of R/M HNSCC patients treated with ICI as a single agent was retrospectively evaluated (ethical committee: NP-3883): 20 patients divided in long-term survivors (LTSs) and short-term survivors (STS), with an OS >18 and <6 months since the start of immunotherapy, respectively (online supplemental etable 2).

The following signatures were evaluated on the Nivactor training dataset and STS-LTS testing set: (1) the Cl-6 subtype classifier described in De Cecco *et al*[9]; (2) 13 GE signatures identified by a literature survey and designed in cohorts of ICI-treated patients or in HNSCC patients (online supplemental etable 3).

Statistics

To evaluate the ability of the Cl-6 subtype to stratify patients based on the survival data, we inspected its distribution by a bivariate kernel density estimation of the joint distribution function fitting the Cl6 signature and the survival variable, retaining the information from censored observations through the funcen function in 'smoothROCtime' R package. Then the Cl6 optimal cut-off value was imputed as the point with the most significant (log-rank test) OS split, and calculated for the entire study population using the "cut-off Finder" tool.[10] A receiver operating characteristic (ROC) curve was used to assess the performance of a binary classifier system. Performance estimates were assessed in terms of: (1) prediction errors, based on Brier scores and (2) discrimination. Univariate Cox's proportional hazards regression was used to analyze the relationship between the biomarker/model and OS and progression-free survival (PFS). We ran a multivariate Cox's regression analysis to see whether biomarkers/models provided more accurate and independent predictions than other covariates. The results are presented in terms of HR and their 95% CIs, along with p values obtained with the two-sided Wald test, and they were assessed using the survival package in R.

RESULTS

Study population

In NIVACTOR study, 124 R/M HNSCC patients were enrolled. According to the primary clinical endpoint, patients experiencing any treatment-related adverse event (TR AE) had an improved OS; HR 0.459 (95% CI 0.254 to 0.827, p=0.0096). Grades 3–4 TR AE happened in 11% of the patients (anemia 2%, pneumonitis and encephalitis 1%), while all-grade TR AE was 40% (fatigue 20%, hypothyroidism 8%, anemia and pruritus 6%). Median duration of grades 3–4 TR AE was 10 days (4–22) and of grade 1–2 TR AE 63 days (1–336). In this phase IIIb trial, safety and efficacy of nivolumab was consistent with previously reported data.[3]

For 80 out of 123 cases, tumor tissue was informatively profiled for GE (online supplemental efigure 1). The Nivactor dataset was considered as training set. No significant differences in gender, age, ECOG (Eastern Cooperative Oncology Group) performance status (PS), smoking, primary tumor site, stage, metastases site and PDL1 status were noted comparing the total cohort and the GE dataset (online supplemental etable 1). In both total and GE cohorts, objective response rate (ORR), according to RECIST criteria (V.1.1), was observed in 122 (98.3%) and 79 (98.7%) patients, respectively; responder patients were 19 in the total cohort (complete responder, CR=2; partial responder, PR=17) and 12 in the GE cohort (CR=0 and PR=12; online supplemental etable 4). The correlation between response status and survival by the

Kaplan-Meier analyses was confirmed for both cohorts (online supplemental efigure 2). The distribution of Cl6 in the 10 patients experiencing grades 3–4 TR AEs was not significantly different) when compared with one of the subjects who did not (p=0.346).

The value of biomarkers such as PD-L1 expression (see online supplemental efigure 1 for number of analyzed cases) and TMB was investigated (see online supplemental efigure 3 for number of analyzed cases). PD-L1 was assessed by Combined Positive Score (CPS; n=93) and Tumor Proportion Score (TPS; n=94); a significant association was found for CPS (negative, CPS<1 vs positive, CPS≥1) with OS as an endpoint (HR=1.753, 95% CI (1.014 to 3.024); p=0.0418), while no significant association was observed between CPS and PFS as well as when TPS (negative, TPS<50 vs positive, TPS≥50) is considered with OS and PFS as endpoints and when CPS and TPS are stratified based on ORR and DCR (online supplemental efigure 4). TMB was assessed in 63 cases and 7 cases showed high TMB status (≥10 mut/Mb). With the caveat of a small sample size (TMB was high in 7 cases), no association was found with OS, PFS, ORR, and DCR (online supplemental efigure 5).

Cl6 signature for predicting prognosis

To identify a GE model/signature with OS as the main clinical endpoint, first we applied the Cl6 HNSCC subtype to the training set. A score indexing all 80 cases was generated. To evaluate the Cl6 distributions and shapes, a kernel density estimation of the joint distribution function of the biomarker and the elapsed survival time was assessed. The density distributions detected two separated groups highlighting different clinical behaviors (figure 1A). The best-dividing threshold by cut-off finder tool was set at 0.06364 value, which stratified in 16 (20%) and 64 (80%) cases (high scores and low scores,

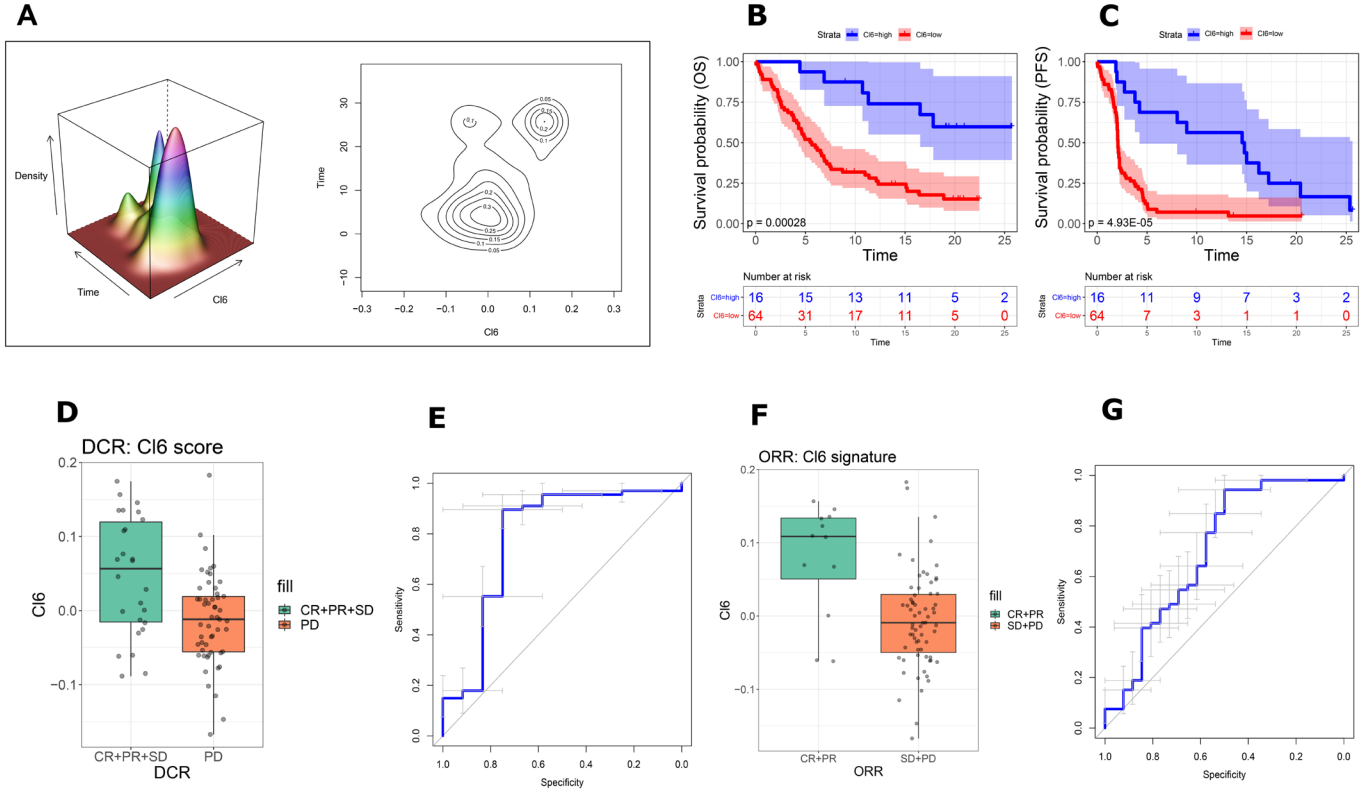

Figure 1 Cl6 signature in the training set. (A) Joint density estimation for the Cl6 signature and survival time variable in the training set visualized at 3D level (left panel) (x-axis=Cl6; y-axis=log(survival time); z-axis=density kernel estimate). Contour plot obtained for the previously considered data (right panel) (x-axis=Cl6; y-axis=log(survival time); the lines indicates iso-values corresponding to 0.05 increase in density kernel estimates). (B) Kaplan-Meier survival curves for patients stratified with high (blue, n=16) or low (red, n=64) Cl6 scores. High-score patients had a longer OS (overall survival) than those with low scores (p=0.00028; HR=4.34, 95% CI 1.84 to 10.22). High-score and low-score curves were compared with the long-rank test. Shadows indicate upper and lower 95% CIs. (C) Kaplan-Meier survival curves for patients stratified with Cl6 high scores (blue, n=16) or low scores (red, n=64). High-score patients had a longer PFS (progression-free survival) than those with low-scores (p=4.93E-05; HR=3.71, 95% CI 1.92 to 7.18). High-score and low-score curves were compared with the long-rank test. HR=HR ratio. Shadows indicate upper and lower 95% CIs. (D) Cl6 and ORR Boxplot of the Cl6 signature for ORR (PR, n=12) and non-responder (SD, n=14; PD, n=53). (E) Cl6 and ORR receiver operating characteristics (ROC) curve and area under the curve (AUC) of the Cl6 signature based on ORR. AUC=0.785 (95% CI 0.603 to 0.967). Gray bars represent 95% CI. (F) Cl6 and DCR. Boxplot of the Cl6 signature for DCR (clinical benefit: PR, n=12 and SD, n=14 vs PD, n=53). (G) Cl6 and DCR. ROC curve and AUC of the Cl6 signature based on DCR. AUC=0.702 (95% CI 0.566 to 0.838). Gray bars represent 95% CI. ORR, objective response rate; PD, progressive disease; PR, partial responder; SD, stable disease.

respectively) and no differences were recorded in demographic and clinical parameters among the two groups (online supplemental etable 5). The patients expressing the Cl6 high scores showed a significantly longer OS than those with Cl6 low scores (log-rank test, p=0.00028; the median OS for high-score and low-score groups was >25 and 5.46 months, respectively (figure 1B). When the Cl6 scores were plotted against OS, high Cl6 scores were associated with HR<1 (online supplemental efigure 6).

Successively, we evaluated the Cl6 ability to stratify patients according to PFS. Kaplan-Meier curves showed a significantly different PFS for the two Cl6 groups (log-rank, p=4.93E−05, figure 1C), corresponding to a median PFS of 14.54 and 2.07 months (Cl6 high-score and low-score groups, respectively).

ORR data analyses were performed by comparing responders (PR; n=12) vs non-responders (stable disease and progressive disease, SD and PD, respectively; n=67). As a continuous variable, the Cl6 signature exhibited significantly higher values in responders than in non-responders (p=0.000171; figure 1D). The model discriminative ability reached an area under the curve (AUC) of 0.785 (95% CI 0.603 to 0.967; figure 1E). When DCR is considered, patients achieving a clinical benefit (CR+PR+SD) showed higher Cl6 signature values than PD subjects (p=0.000491; figure 1F), reaching AUC of 0.702 (95% CI 0.566 to 0.838; figure 1G).

Performance of Cl6 signature

Discrimination analysis of the Cl6 based on OS was assessed by a time-dependent ROC curve, accounting the censoring pattern of patients over the whole follow-up period (figure 2A). The AUC at the landmark follow-up times of 3, 6, 12, and 18 months had a value of 0.633 (95% CI 0.577 to 69), 0.659 (95% CI 0.582 to 0.737), 0.667 (95% CI 0.563 to 0.771), 0.706 (95% CI 0.571 to 0.841). Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) at the landmark times are detailed in online supplemental etable 6.

To test the validity of the model, the Cl6 prediction error in fitting the survival information was examined with the Brier score. The results showed that the expected Brier score was lower than the reference scenario's score when it took the risk identified by the signature into account. As a summary measure of the Brier scores, the cumulative prediction error (IBS) over an interval ranging from 3 to 18 months yielded 0.232, 0.22, 0.194, and 0.19 for the reference scenario, PS, Cl6 signature, and a model integrating Cl6 and PS, respectively (figure 2B).

The assessment of time-dependent ROC and prediction error for Cl6 and PS in terms of PFS is reported in online supplemental efigure 7.

Univariate and multivariate regression models were used to assess the prognostic value of the Cl6 compared with other clinical features (ECOG PS, PDL1 CPS, and tumor primary site). Univariate analysis, with OS as endpoint, indicated that Cl6 and PS correlated significantly with survival. When all the covariates were analyzed in a multivariate model, only the Cl6 retained a significant association. When PFS was considered, only Cl6 was significantly associated at univariate and multivariate analyses (table 1).

Discriminative ability of Cl6 signature to identify LTSs

To challenge our Cl6 signature's performance, the independent cohort of 20 platinum-resistant RM-HNSCC patients treated with ICI alone divided in LTS and STS was used as testing data set (online supplemental etable

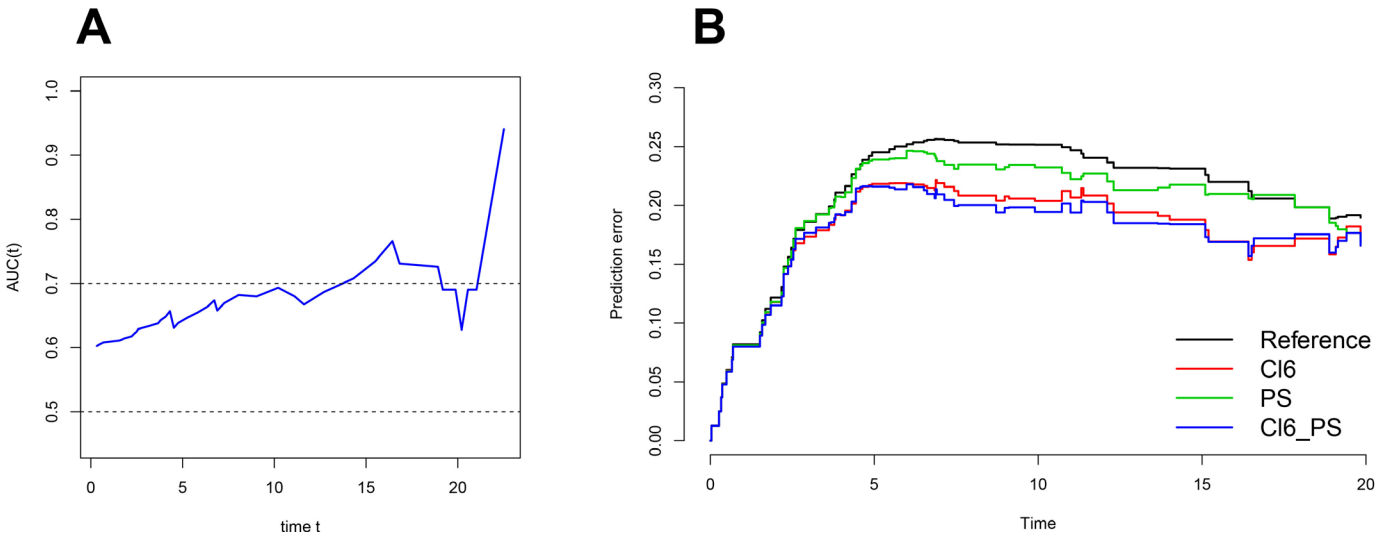

Figure 2 Performance of Cl6 signature. (A) Area under the ROC curves (AUC) based on Cl6 signature fitting the OS, obtained from a time-dependent ROC analysis. (B) Prediction error curves by Brier scores. The Cl6 signature, ECOG_PS, and an integrated model with Cl6 and ECOG_PS were compared with estimates for all patients without any stratification applied (reference scenario curve). For a single patient, the Brier score at the time t is defined as the squared difference between the observed survival status and the predicted outcome probability. ECOG, Eastern Cooperative Oncology Group; OS, overall survival; PS, performance status; ROC, receiver operating characteristic.

Table 1 Results of Cox's proportional hazard regression analysis

	Univariate analysis (OS)		Multivariate analysis (OS)	
OS	**HR (95% CI)**	**P value**	**HR (95% CI)**	**P value**
CI6 (low vs high score)	3.71 (1.918 to 7.175)	**7.97E−04**	4.576 (1.782 to 11.752)	**0.00158**
ECOG PS (1 or 2 vs 0)	2.188 (1.19 to 4.02)	**0.0117**	1.785 (0.933 to 3.414)	0.08011
PD-L1 (CPS<1 vs ≥1)	1.731 (0.938 to 3.195)	0.0793	1.673 (0.863 to 3.242)	0.12761
Primary disease site (other vs oropharynx)	1.403 (0.739 to 2.665	0.301	1.007 (0.508 to 1.996)	0.98339
	Univariate analysis (PFS)		**Multivariate analysis (PFS)**	
PFS	**HR (95% CI)**	**P value**	**HR (95% CI)**	**P value**
CI6 (low vs high score)	4.337 (1.84 to 10.22)	**9.79E−05**	3.624 (1.782 to 7.37)	**0.00038**
ECOG PS (1 or 2 vs 0)	1.648 (0.992 to 2.739)	0.0539	1.416 (0.824 to 2.433)	0.208
PD-L1 (CPS <1 vs ≥1)	1.413 (0.804 to 2.484)	0.23	1.491 (0.816 to 2.722)	0.194
Primary disease site (other vs oropharynx)	1.44 (0.822 to 2.522)	0.202	1.104 (0.612 to 1.993)	0.743

P values <0.05 are in bold.
ECOG PS, Eastern Cooperative Oncology Group performance status; OS, overall survival; PFS, progression-free survival.

2). The CI6 signature was able to stratify patients with significantly higher values in LTS (n=12) than in STS (n=8) (p=0.00544; figure 3A). The discriminative ability of the GE reached an AUC=0.864 (95% CI 0.687 to 1; figure 3B).

GE signatures

Thirteen signatures were retrieved from literature including: (1) six immune-related signatures associated to ICI outcome and whose predictive or prognostic value was validated in clinical studies with ICI treatment on different tumor types[11–14] (online supplemental etable 3A) and (2) seven signatures related to immune proprieties of HNSCC, developed on TCGA data and with a prognostic impact on ICI untreated patients[15–22] (online supplemental etable 3B). We assessed the Pearson's correlation among these signatures and CI6, proving that IFNg, Expanded, Inflammation, Immune, and T-cell Inflammed signatures significantly cross-correlate (r>0.8, p<0.0001), while CI6 shows an indirect correlation with Hu_2021 signature (r=−0.414, p=6.72E−05)

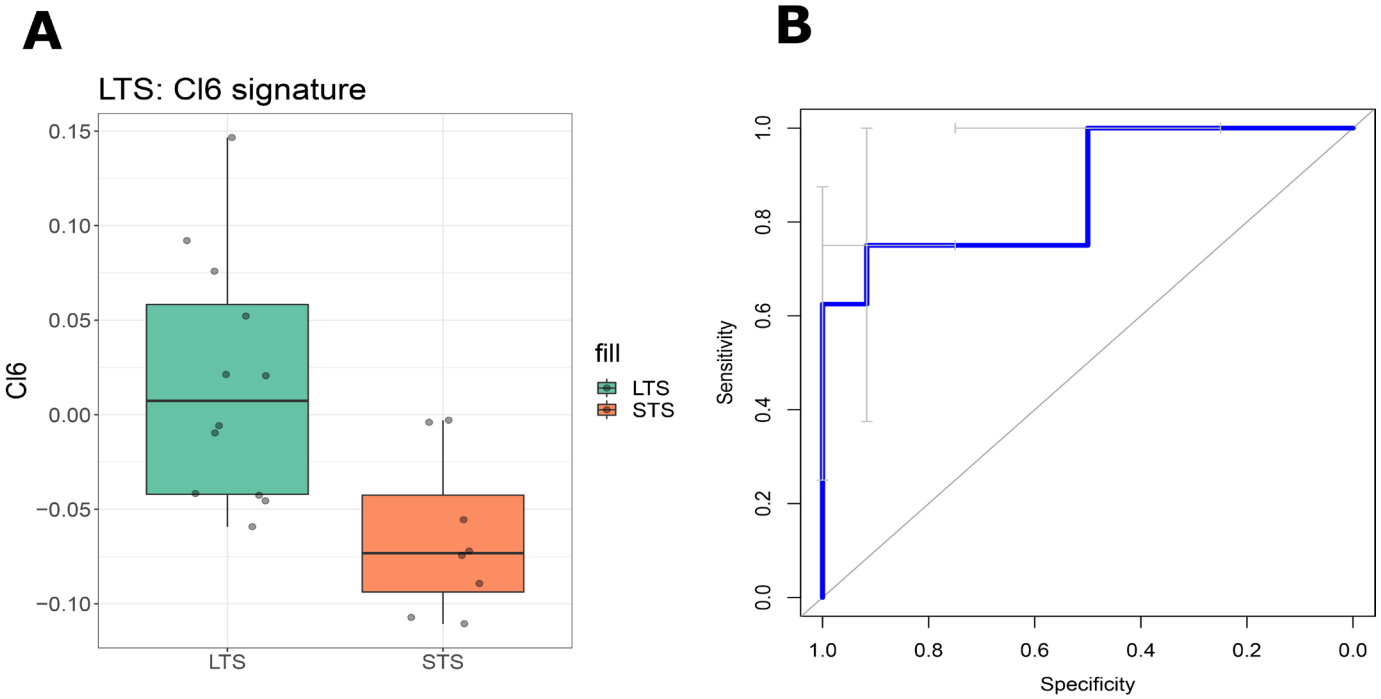

Figure 3 Stratification ability of CI6 signature in the test set. (A) Boxplot of the CI6 signature in LTS and STS cases. Median value is significantly higher in LTS with broader IQRs compared with STS (p=0.00544). (B) Receiver operating characteristics analysis and area under the curve (AUC) of the CI6 signature. AUC=0.864; (95% CI 0.687 to 1). Gray bars represent 95% CI. LTS, long-term survivor; STS, short-term survivor.

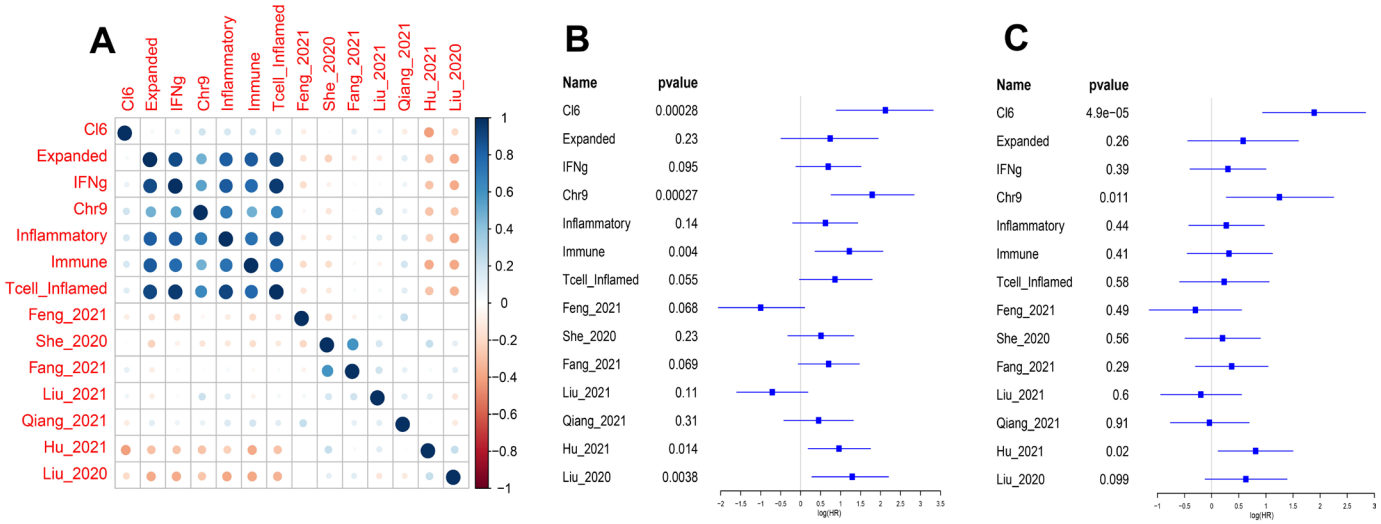

Figure 4 Immune-related signatures on Nivactor. (A) Correlation plot. The plot depicts the Pearson correlations of signature pairs in a colorimetric scale. The size of each point corresponds to the magnitude of the correlation. To ascertain if unseen dependencies are present in our data, Spearman correlation was also evaluated (online supplemental efigure 12). (B) Forest plot for OS of the 13 signatures compared with C6. Patients were stratified based on high-score and low-score signature and associated to OS as clinical endpoint. (C) Forest plot for PFS of the 13 signatures compared with Cl6. Patients were stratified based on high-score and low-score signature and associated to PFS as clinical endpoint. OS, overall survival; PFS, progression-free survival.

(figure 4A). Based on high versus low signature ratio, Chr9 (log (HR)=−1.79, 95% CI (−1.79 to −2.84); p=0.00027), Immune (log (HR)=−1.22, 95% CI (−2.06 to −0.36); p=0.004), Hu_2021 (log (HR)=0.96, 95% CI (0.19 to 1.75); p=0.014), Liu_2020 (log (HR)=1.29, 95% CI (0.28 to 2.2); p=0.0038) showed a significant association to OS (figure 4B); while only Chr9 (log (HR)=−1.25, 95% CI (−2.25 to −0.27); p=0.011) and Hu_2021 (log (HR)=0.81, 95% CI (0.12 to 1.5); p=0.02) confirmed a significant association with PFS (figure 4C). The Kaplan-Meier plots are reported in online supplemental efigure 8. When the signatures were challenged in the test set, IFNg, Inflammed, Chr9, and T-cell Inflamed signatures were found significantly (p<0.05) associated with higher values in LTS than in STS (online supplemental efigure 9). The association for the 13 investigated signatures with ORR was evaluated in our NIVACTOR cohort and no significant differences were found (online supplemental efigure 10).

DISCUSSION

With the advent of ICIs in cancer treatment, different biomarkers were investigated, such as PD-L1 (using distinct scoring systems), and TMB to stratify patients and select efficient treatment options. Recently, evidence-based recommendations pointed out the importance in testing both PD-L1 (especially considering CPS) and TMB in ICI-treated R/M HNSCC patients.[23] However, the predictive ability of these biomarkers against the response to ICI and patient's survival is still limited. The Nivactor study mirrors this observation, with some degree of predictive accuracy for PD-L1 and TMB, where a significant association with OS was recorded only for CPS (≥1) but not confirmed when PFS or response were tested, while TPS and TMB were associated neither with survival nor with response. Overall, the present available data underlie the need to explore other biomarkers able to better define the patients with the highest benefit from ICI.

Therefore, in order to improve patient stratification, we tested the prognostic/predictive value of immune-associated gene model/signatures. Our approach was based on evaluation of already generated signatures to verify their validity and performance using an independent well-defined clinical dataset. Immune-related model (1: Cl6) and signatures (13) were retrieved from literature and assessed in testing (Nivactor) and validation (STS-LTS) GE datasets. In both cohorts, the Cl6 model, (ie, the immune-reactive Cluster6 subtype), identified patients with significantly better response and longer OS and PFS. Additionally, according to Brier score, Cl6 proved to ameliorate stratification over other signatures, biomarkers and clinical features (ie, ECOG PS). The Cl6 was one of the six molecular clusters identified by De Cecco *et al* through a robust HNSCC subtype classification (doi:10.18632/oncotarget.3301), whose principal characteristics were: (1) stratification based on primary tumors; (2) cluster identification trough a deconvolution approach assessing the deviation of each tumor from the normal phenotype; (3) use of a large meta-analysis composed by multiple HNSCC datasets (training: 8 studies, 527 cases; validation: 12 studies, 859 cases). Specifically, in its original designation Cl6 model showed the higher similarity to the normal state and activation of several immune-related pathways including IFN-I. Moreover, the Cl6 model expressed a correlation with good

prognosis (second only to HPV-like Cl1 subtype). Similarly, another cluster defined in the De Cecco subtyping analysis[9] (ie, Cl3 "hypoxia") demonstrated a relevant clinical value when challenged against HNSCC patients treated with EGFR-inhibitors,[24–27] confirming the potential role of this subtypes classification when tested against specific oncologic treatments. Here, the reported data confirmed the strong prognostic role of Cl6, and for the first time, suggested its possible implication in predicting the ICI response in R/M HNSCC patients.

When we analyzed the six immune-related signatures developed/tested in ICI-treated patients from clinical trials, they resulted strongly cross-correlated but only the Chr9 signature significantly identified patients with shorter survival in both Nivactor and STS/LTS dataset. Notably, the Chr9 signature,[28] following the evaluation in original paper, was challenged in a real-world cohort of PD-1 inhibitors treated HPV–HNSCC patients confirming its prognostic role. The 9p21-loss status was identified as an independent genomic biomarker associated with decreased OS during PD-1 inhibitors treatment.[29] The numerous lost genes were associated to many pathways including those regulated by IFN-I and very recent experimental data seem to confirm the role of alteration in IFN-I as a pervasive genetic mechanism of immune evasion in cancer. Our findings proved that Cl6 survival performance is improved when combined with Chr9; however, Chr9 fails in adding a significant value on predicting progression events over time.

The role of the other five ICI-associated signatures was limited: the Immune signature significantly associated to longer OS only in the Nivactor dataset; three signatures (IFNg, inflammatory, T-cell inflammed) were only able to significantly separate STS from LTS patients in the validation set.

The seven immune-related signatures were all generated using TCGA HNSCC dataset and by selecting tumor microenvironment genes associated to immune-suppression or cold-immune status; accordingly, in the original papers all are associated to poor prognosis. In agreement with their "specific generation", these immune-related signatures exhibited a trend of inverse-correlation with the ICI outcome signatures and in particular, Hu-2021 signature was also slightly inverse-correlated to Cl6. Only two immune-related signatures (Hu-2021, Liu-2020) were associated to worse OS in Nivactor and only Hu-2021 signature was also associated to worse PFS; none of these signatures was associated to LTS-STS survival.

The major strength of the GE model (Cl6) and signature (Chr9) we identified relies on their performance, able to overcome any other biomarkers, as well as the clinical prognostic factors employed in RM HNSCC, as PS and tumor subsite. The Cl6 model could be used in identifying the small subgroup of patients benefiting from ICI when resistant to platinum-based chemotherapy and the Chr9 signature in selecting the small subgroup of patients who should not be treated with ICI, the remaining patients being candidates for clinical trials with new agents to revert primary resistance to ICI. In this regard, a deeper biological analysis of the immune signatures with opposite directions might be used to better tailor therapeutic interventions in this group of patients, suggesting the pathways to be tackled. The majority of study patients progressing on nivolumab (73.6%, data not shown) did not receive any subsequent medical treatments. Therefore, we hypothesize that the contribution subsequent treatments on the prognostic analyses should be limited.

Our effort offers novel insights on the clinical relevance of gene signatures in R/M HNSCC patients treated with ICI. As ICI treatment is now adopted also for platinum-sensitive diseases, either as monotherapy or in combination with chemotherapy in first-line treatment for R/M HNSCC, the logical next step is studying the Cl6 model and Chr9 signature also in this setting of patients. This will allow to elucidate their performance also in an earlier setting of disease and to verify the role of Cl6 when chemotherapy is added to ICI.

Limitations are the lack of PD-L1 and TMB data for a fraction of patients, the absence of CRs in the analyzed GE dataset. Another limitation is the not available access to GE datasets of other ICI HNSCC clinical trials. Moreover, the number of cases included in the study is relatively small. Thus, before promoting this GE signature as a widely used predictive indicator for R/M HNSCC patients receiving immunotherapy, further validation is needed, for instance in the first-line platinum-sensitive setting.

Nonetheless, the results of this study suggest that Cl6 model may be a valuable prognostic, and possibly also predictive, biomarker for estimating long survival in platinum-resistant ICI-treated R/M HNSCC patients. In addition, the application of Cl6 model to wider series of patients treated with ICI, the possible integration with Chr9 signature and investigation of biomarkers in liquid biopsies may better define the subset of patients who may benefit of ICI in term of response and long survival. Also, this prognostic signature could help identifying the group of patients who could benefit from other therapeutic intervention instead of ICI or to be added to ICI in order to reverse an immunosuppressive microenvironment. The application to Cl6 signature to first line, platinum-sensitive RM HNSCC patients needs to be elucidated by future research.

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Ethics approval The Nivactor ("A Single-Arm, Open-Label, Multicenter, Phase IIIb Clinical Trial with NIVolumab in Subjects with Recurrent or Metastatic Platinum-refrACTORy Squamous Cell Carcinoma of the Head and Neck") study, a prospective phase IIIb single-arm multicenter clinical trial (EudraCT Number: 2017-000562-30) was conducted in accordance to ethical guidelines and regulations and approved by the local ethical committee (INT128/17). The patients provided written informed consent.

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Data availability statement Data are available in a public, open access repository. Microarray data were compliant to MIAME (Minimum Information about a Microarray Experiment) and the GE data of our cohorts were deposited on GEO (accession number GSE212551). Detailed methods including PD-L1 evaluation, nucleic acid extraction, GE profiling, signatures performance, bioinformatic tools, statistical analysis and NGS experiments are reported in online supplemental eappendix.

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